THE ACTION OF CHOLINE AND RELATED COMPOUNDS ON THE HEART OF VENUS MERCENARIA [1]

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Since the demonstration by Prosser (1940) of the unusual sensitivity to acetylcholine (Ach) of the isolated heart of the bivalve molusc, *Venus mercenaria,* we have extensively employed this preparation for the bio-assay of Ach in tissue extracts (e.g. Welsh, 1943; Welsh and Hyde, 1944a and b; Prajmovsky and Welsh, 1948). In certain respects it is superior to the classical Ach assay preparations such as the dorsal muscle of the leech, rectus abdominis of the frog, isolated frog heart, and blood pressure of cat. For example, it is more sensitive to Ach, with complete inhibition occurring at about 50 times the threshold inhibitory concentration; it is relatively unaffected by changes in pH, inorganic ions, and tissue constituents other than Ach; it recovers quickly, thereby allowing more rapid estimation than the above-mentioned preparations.

While employing the *Venus* heart for bio-assay, its responses to a variety of drugs, organic compounds, and inorganic ions have been studied and, in particular, to a series of choline esters and analogs—this in the hope of obtaining evidence toward a better understanding of the fundamental mode of action of Ach. An organ with a high specificity for choline esters, exhibiting a response which is easily quantified, and which has so little self-contained cholinesterase that blocking of this enzyme is not necessary when working at great dilutions of the unstable esters, provides a suitable object for studying certain aspects of the mechanism by which Ach acts on cells.

The present paper has two purposes: (1) to indicate the methods of preparing and employing the *Venus* heart for the bio-assay of Ach, and (2) to compare the effects of other choline esters and related compounds which differ in greater or less degree from the Ach molecule.

Methods of Preparing and Using the Hearts for Ach Estimation

An earlier paper by Wait (1943) covers some of the necessary procedures for preparing the isolated *Venus* heart for Ach determinations. For convenience, however, the steps which we employ from the securing of appropriate test animals to the quantitative estimation of Ach in tissue extracts will be outlined.

Venus mercenaria (the hard shell clam or quahog), being an important commercial shellfish along the Atlantic Coast, are usually available where shellfish are sold. They remain edible for some weeks after digging or dredging, if maintained under refrigeration, but after one to two weeks the hearts of such animals tend to beat with an irregular rhythm; it is important, therefore, to obtain experimental

[1] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

material from a source where the previous history is known [2] and to use this material within one to two weeks after removal from the sea. *Venus* with a shell length of 8 to 12 cm. have been found of most convenient size. In the laboratory they may be stored dry at 5°–10° C., or preferably kept in shallow tanks of aerated sea water, at 15°–18° C.

The heart is exposed by breaking and removing the dorsal portion of the shells (umbos and hinge) and then cutting away the mantle and pericardium dorsal to the heart. The heart consists of a single, median ventricle with laterally disposed, thin-walled atria (auricles). Anterior and posterior blood vessels leave the heart in close association with the intestine which passes through the heart. Threads for attaching to a support in the bath and to the writing lever may be passed under the atria and tied close to the ventricle in order to include some of the thicker-walled, ventricular muscle. Cutting the atria distal to the threads, and cutting the blood vessels and intestine, isolates the ventricle which may then be placed in an appropriate heart bath. Only the outer surface of the heart is directly exposed to materials introduced into the bath, but cannulation of the heart and introduction of Ach into the ventricle does not increase its sensitivity.

The bath figured by Wait (1943) is satisfactory unless temperature control is desired (e.g. when working in a room above 20° C., or when maximum sensitivity is required); then a bath with a water jacket through which water of an appropriate temperature (15°–18° C.) is circulated may be employed, or the heart bath may be placed in a larger temperature-controlled vessel. It is necessary that provision be made for changing the fluids of the heart bath without draining the bath and subjecting the heart to undue mechanical disturbance. A bath holding 10 ml. when filled has been found appropriate.

An analysis of the inorganic salts of the blood of *Venus mercenaria* by Cole (1940) showed only small differences in comparison with sea water. It is not surprising, therefore, that sea water is an adequate perfusion fluid for the isolated heart, allowing a regular beat to be maintained for 2–3 days. Where natural sea water is not available, an artificial perfusion fluid may be used and several have been tried, with differing ratios of the common ions, without noting any appreciable effects on the heart until radical departures from the normal concentrations of the common ions are made. A fluid found satisfactory has the following composition: 30 gm. NaCl; 0.9 gm. KCl; 1.1 gm. $CaCl_2$; 3.5 gm. $MgSO_4 \cdot 3H_2O$ in one liter of water with a phosphate or bicarbonate buffer (pH 7–7.5). Changes of pH between 6 and 8.5 have little or no effect on the amplitude or frequency of beat, or on the response of the heart to acetylcholine for periods of time up to several hours.

Oxygen may be supplied by air or a mixture of 95 per cent O_2–5 per cent CO_2 passed through the bath. The bubbles should be small to avoid mechanical disturbance to the heart. The gas mixture or air may be admitted to the bath through the hooked support for the lower attachment of the heart if this is made from glass tubing drawn out to a fine tip. The heart lever should be counterweighted to give a pull of 200–300 mg. A kymograph speed of about 2 cm. per minute is desirable. Substances to be tested may conveniently be added at the bottom of the bath by

[2] E.g. Supply Department, Marine Biological Laboratory, Woods Hole, Mass., or a wholesale dealer in shellfish.

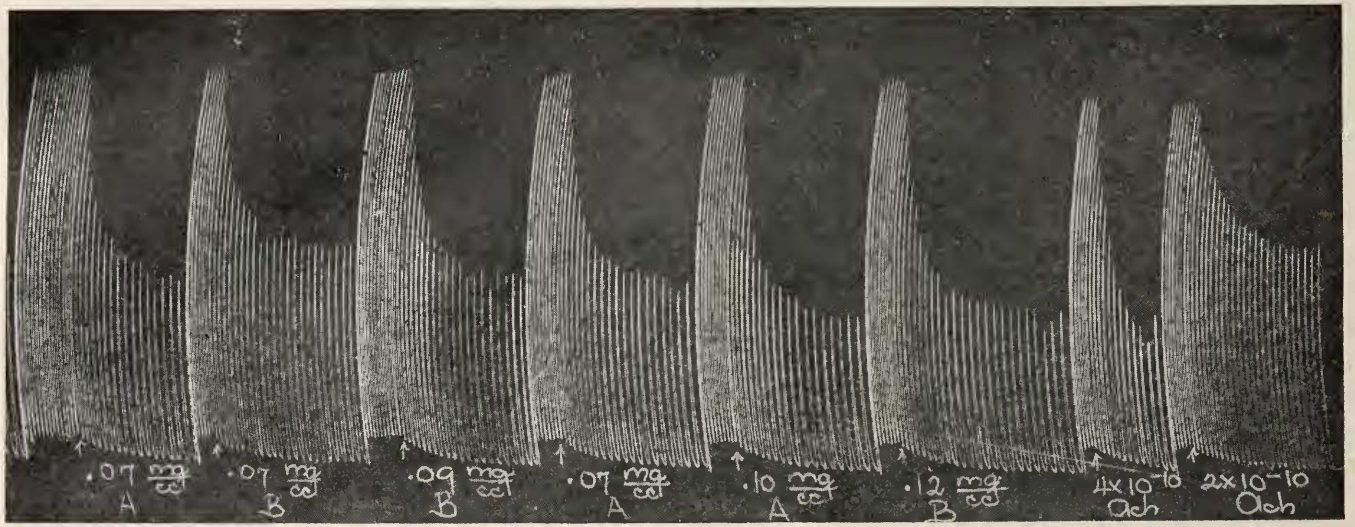

FIGURE 1. Sample record of bio-assay using the isolated *Venus* heart to estimate the amounts of extractable acetylcholine in two isolated ventral nerve cords (A and B) of nymphs of the cockroach, *Blaberus*. Total wet weights of tissue available: cord A = 17.3 mg.; cord B = 15.4 mg. The sensitivity of this heart to Ach and the approximate activities of the extracts had been determined in an earlier portion of the record. At the first arrow an amount of extract of cord A was added to the bath to give a final dilution of substance per cc. yielded by an original wet weight of tissue of 0.07 mg. The extract remained in the bath approximately one minute; the drum was stopped and the fluid of the bath changed several times and the heart allowed 3–5 min. to recover its original amplitude. After recording a few normal beats the extract of cord B was added at the second arrow. Following two attempts to determine amounts of extracts of cords A and B required to produce equivalent decreases in amplitude of beat, two known concentrations of Ach were applied (4×10^{-10} and 2×10^{-10} gm./cc.). From the results it was possible to calculate that cord A contained an Ach-like material equivalent to 4.0 μg. Ach/gm. wet weight while cord B yielded a value of 3.3 μg. Ach/gm. On a second heart the values obtained were A = 5.0 μg. Ach/gm.; B = 4.2 μg. Ach/gm.

means of a long hypodermic needle bent at a right angle; the volumes added should be small (1 ml. or less).

In estimating the Ach content of a tissue extract, a dilution should be found that gives between 20 and 80 per cent decrease in amplitude of beat at the end of one or two minutes. This should be matched, preferably twice, with known concentrations of Ach, after which appropriate calculations will give the Ach equivalent per gram of tissue. A sample record is shown in Figure 1. If it is suspected that substances in the tissue extract other than Ach are affecting the heart, it may be desirable to treat a portion of the extract by the addition of NaOH and warming in order to destroy the Ach present, and after neutralizing with HCl, to use this to make up the last dilution of Ach prior to adding to the bath.

Treatment of a heart with an anti-cholinesterase (physostigmine, neostigmine or di-isopropyl fluorophosphate) may potentiate the action of Ach two to five times. This small degree of potentiation is undoubtedly due to the low level of cholinesterase activity in these hearts (Smith and Glick, 1939; Jullien et al., 1938). Because the untreated heart is so sensitive to Ach (threshold for inhibition is usually between 10^{-10} and 10^{-11} gm. per ml.), and because recovery after Ach is slowed by treatment with an anti-cholinesterase, it is normally undesirable to employ this means for increasing sensitivity.

Occasionally hearts are encountered which fail to beat or which beat with a low amplitude. Although adrenalin and tyramine have been found to be excitants in relatively high concentrations (5×10^{-5} to 10^{-4} M) their effects are quickly abolished by washing. On the other hand ergotoxine, ergotamine, and ergonovine have been found to have a remarkably persistent excitatory action. For example, one part per million of ergotoxine ethanesulfonate will frequently cause renewal of heart beat, or an increase in amplitude of two to three times, in a heart with an abnormally low amplitude. Treatment for a few minutes with one of these ergot alkaloids produces a change in the physiology of the heart which persists for many hours in spite of repeated washings, while the response to Ach is affected but slightly. In this connection it should be noted that the *Venus* heart is composed of smooth muscle and its pharmacology is not unlike that of certain types of vertebrate smooth muscle.

There is some seasonal variation in the sensitivity of *Venus* hearts to Ach, with maximum sensitivity in the late winter and spring months (cf. Prosser, 1940; Wait, 1943), but the change is probably not as great as indicated by Prosser. More important to note is that in late summer there is a tendency toward irregularity in beat. This is often so pronounced that accurate estimates of small differences in Ach levels cannot readily be made in August and early September in the region of Massachusetts.

The Action of Certain Choline Derivatives and Related Compounds on the Venus Heart

The greatest gap in our knowledge concerning Ach is the precise manner in which this physiologically active substance affects the excitability of cells. More detailed studies of the mechanism of action of Ach are needed, and it would appear to matter little what type of Ach-sensitive tissue or organ is used in these studies. For many reasons the isolated heart of the quahog appears to be peculiarly suitable

for such a study, and in this section of the present paper it will be shown that no compound related to Ach has yet been found having as great an inhibitory action on this organ as does Ach. It will also be shown that the methyl grouping around the onium element is, in many respects, the most significant portion of the Ach molecule.

The typical effects of Ach on the *Venus* heart will first be described, and then the relative activities of a number of common choline derivatives and certain related compounds will be discussed.

When Ach is added to a bath containing a beating *Venus* heart, which has received no previous drug treatment, to give a concentration in the vicinity of 10^{-11} to 10^{-10} M, a small increase in amplitude is sometimes observed. A similar stimulating action of low concentrations of Ach on vertebrate hearts has been observed by McDowall (1946) and others. At concentrations of Ach in the vicinity of 10^{-9} to 10^{-8} M a negative inotropic effect is seen; and with increasing amounts of Ach the amplitude of beat decreases until the heart stops in diastole at a concentration of Ach about 50 times that which gives a just measurable decrease in amplitude. Thus the range of concentrations from the threshold of inhibition to complete inhibition is relatively narrow. The log-concentration-response curve is sigmoid, with the portion between 20 and 80 per cent inhibition approximating a straight line. The inhibitory action of Ach on the *Venus* heart is more prominent and consistent than the excitatory action of lower concentrations, but it seems probable, as pointed out elsewhere (Welsh, 1948), that Ach first excites and then in higher concentrations inhibits or paralyzes this organ as it may do to all tissues or organs which respond to Ach.

In Table I a summary is given of the relative inhibitory activities of a number of compounds related to choline or Ach. The data shown in this table were obtained by finding a molar concentration of Ach that would produce between 20 and 80 per cent decrease in amplitude of beat of a given heart, and then the molar quantity of a related compound that would produce a degree of inhibition exactly matching that produced by the Ach. Each value shown for a given compound was obtained on a different heart. The average values may be taken as a fairly precise indication of the relative inhibitory effectiveness of these several compounds on the *Venus* heart. Since cholinesterase activity in this organ is extremely low, anti-cholinesterases were not employed, but the complications which may arise when stable choline esters are compared with unstable in the presence of active cholinesterases are believed to be minimal.

In commenting on certain of the more interesting facts given in Table I attention may first be called to choline. Choline affects the isolated *Venus* heart in a manner very much like that of Ach, except that it is far less active. When first applied in low concentrations, the amplitude of beat may increase (Fig. 2, Curve 1). Greater variation in the response of different hearts to choline was observed than in the case of any other compound. This is illustrated by Figure 2, where concentration response curves for three different hearts are shown. The wide range of values obtained when choline was compared with Ach may be accounted for by individual variation in the response to choline, for it is obvious that if a match of molar concentrations of Ach and choline producing 25 per cent inhibition were made on the heart represented by curve 3, the relative value for choline might be 1000; while a match of 25 per cent inhibition made on the heart represented by curve 2 would yield a value showing Ach to be perhaps 50,000 times as active as choline.

TABLE I

Relative molar quantities required to produce a decrease in amplitude of beat equivalent to that produced by a molar quantity of acetylcholine chloride equal to 1

Choline derivative	Structural formula	Molecular weight	Values obtained	Averages
Carbamylcholine chloride	$(CH_3)_3NCH_2CH_2OCNH_2$ (Cl; O)	182.5	20 20 25 40 40 50 80 100 100 150 250	80
n-Propionylcholine chloride	$(CH_3)_3NCH_2CH_2OCCH_2CH_3$ (Cl; O)	195	100 100 100 120	105
Ethoxycholine bromide	$(CH_3)_3N—CH_2CH_2OCH_2CH_3$ (Br)	212	100 100 100 130 200 200 200 250	160
Butyrylcholine chloride	$(CH_3)_3N—CH_2CH_2OC(CH_2)_2CH_3$ (Cl; O)	209	200 500 800 1,000	625
Chloracetylcholine chloride	$(CH_3)_3NCH_2CH_2OCCH_2Cl$ (Cl; O)	215.9	500 750 1,000 1,600	960
Acetyl β methyl choline chloride	$(CH_3)_3N—CH_2CHOCCH_3$ (Cl; CH_3; O)	195	400 1,000 1,000 2,000	1,100
Benzoylcholine chloride	$(CH_3)_3NCH_2CH_2OC—C_6H_5$ (Cl; O)	243	10,000 10,000 12,000 13,000 20,000	15,000
Choline chloride	$(CH_3)_3NCH_2CH_2OH$ (Cl)	139.5	400 1,000 1,000 1,000 1,000 2,000 4,000 5,000 7,000 15,000 40,000 100,000	14,000
Betaine ethyl ester chloride	$(CH_3)_3NCH_2COCH_2CH_3$ (Cl; O)	181.5	1,000 1,000	1,000
Betaine hydrochloride	$[(CH_3)_3NCH_2CO]$ (O) $\cdot HCl$	117	3,300 5,000 10,000 20,000 25,000 25,000	15,000
Triethylcholine chloride	$(C_2H_5)_3NCH_2CH_2OH$ (Cl)	272	10^{-2} M—no inhibition	
Triethylacetyl choline iodide	$(C_2H_5)_3NCH_2CH_2OCCH_3$ (I; O)	315	10^{-2} M—no inhibition	

However, the average value showing that 14,000 times as many molecules of choline are required than of Ach to produce a given effect, is probably a close approximation to the relative activities of these two compounds on the *Venus* heart. Thus the acetic acid ester of choline is far more active than is the parent compound. This is an observation that has been made by many workers on many tissues and organs.

The several esters of choline which were tested and the one ether (ethoxycholine) were all far more active than choline, with the exception of benzoylcholine which has approximately the same level of activity. The presence of the ring structure at the non-polar end of the molecule obviously affects the activity greatly. It is of interest to note that the substitution of chlorine for a hydrogen atom of the terminal methyl group in Ach to yield chloracetylcholine reduces the activity approximately one thousand fold.

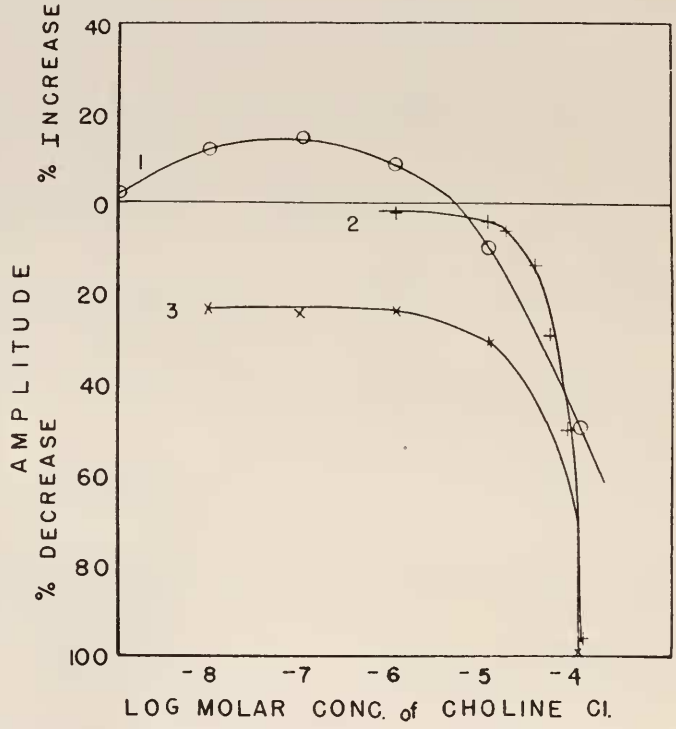

FIGURE 2. Showing the extreme variation in response of three different isolated *Venus* hearts to choline. Heart number 1 responded by an increase in amplitude to low concentrations of choline while hearts 2 and 3 showed only a decrease in amplitude, but their sensitivities differed markedly.

Betaine, a naturally occurring compound closely related to choline, is approximately equivalent to choline in its ability to depress the beat of the *Venus* heart, while the ethyl ester of betaine was found to be considerably more active than betaine.

A clear indication of the importance of the methyl groups attached to the nitrogen was seen when triethylcholine and triethylacetylcholine were tested. In the highest concentrations employed (10^{-2} M), neither of these produced the slightest degree of inhibition of heart beat. We have observed a similar striking difference in the actions of the tetramethyl ammonium ion, which decreases the amplitude of beat of the *Venus* heart, and tetraethyl-, tetra-n-propyl-, and triethyl-n-octyl ammonium

ions, all three having an excitatory action only. These results obtained with the quaternary ammonium ions will be reported more extensively in a separate paper.

Importance of the methyl groups attached to the onium element directs attention to this portion of the Ach molecule. It is apparent from the present study and from similar earlier studies that different choline esters differ in their degree of pharmacological activity. This is also true of the *Venus* heart, but they all produce a characteristic decrease in amplitude. The substitution of ethyl for methyl groups in choline and Ach yields molecules which are completely lacking in inhibitory activity when applied to the isolated *Venus* heart. It has been indicated elsewhere (Welsh, 1948) that this specificity of the $(CH_3)_3N$ group, the rapidity of action of and recovery from Ach, and its activity in small amounts, suggest that Ach acts at the surface of cells as a "trigger" to set off a reaction or chain of reactions in the manner of an unstable coenzyme. Thus, the condition of the cell membrane is altered and the cell excited and then depressed depending on the concentration and time of action of the Ach. In further testing of this hypothesis, it is believed that the isolated *Venus* heart will continue to provide an ideal experimental object.

Summary

1. A method of preparing and employing the isolated heart of the quahog, *Venus mercenaria,* for the bio-assay of acetylcholine (Ach) is described.

2. The activities of choline and certain choline esters; of betaine and its ethyl ester; and of triethylcholine and triethyl-acetylcholine on the isolated *Venus* heart are compared. In further understanding the fundamental mode of action of Ach, the most significant observation was that the substitution of ethyl groups for methyl on the nitrogen of choline and Ach resulted in a complete loss of activity determined by observation on the amplitude of heart beat.

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